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Research Article

MOLECULAR DETECTION OF *LISTERIA MONOCYTOGENES* AND *L. INNOCUA* ISOLATES IN FISH SKIN SWABS FROM RETAIL FISH MARKETS IN WEST BENGAL, INDIA

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ABSTRACT: *Listeria monocytogenes* is a pervasive pathogen that survives well in nature and recognised from various animal food including contaminated fish and fish products worldwide and in India.The presence of *L. innocua,* a non-pathogenic *Listeria*, indicates improper hygienic practices suitable for *L. monocytogenes* contamination in food.To detect contamination by *Listeria innocua* and *monocytogenes* in the skin of freshwater fish rohu (*Labeo rohita*), 234 swab samples were collected from retail fish markets of Kolkata (n=165) and South 24 Parganas (n=69) districts of West Bengal by standard microbiological and molecular biology protocols. One hundred four (44.44%) *Listeria* spp. strains were identified by cultural characteristics, biochemical tests, and PCR technique targeting genus-specific *prs* gene PCR, of which 6 (2.56%) and 11 (4.7%) isolates were detected as *L.monocytogenes* and *L.innocua* targeting the *lmo1030* and *lin0464* genes, by PCR, respectively. The occurrence of *L. monocytogenes* was found less in this study. Regular monitoring systems should be practiced to uphold the quality of Indian food and maintain food safety standards.

Keywords: Fish, Listeria, Labeo rohita, PCR, West Bengal

INTRODUCTION

The management of foodborne diseases presents significant hurdles for developing nations [1]. *Listeria monocytogenes* causes Listeriosis, an intracellular bacterium, occurs in several animals and humans [2]. *L. monocytogenes* is a rod-shaped, non-spore-forming, facultative anaerobic Gram-positive bacteria omnipresent in nature [3]. Survival at low temperature of the refrigerator (2–4°C), salt tolerance (10% NaCl), and growth in variable pH conditions (pH 4.5–9) mark their efficiency in environmental existence [4]. Recently more than 20 species have been distinguished under the genus *Listeria*, of which the *Listeria sensu stricto* group consisted of *L. monocytogenes*, *L. welshimeri, L. innocua, L. seeligeri, L. ivanovii, L. grayi* [5]. Among these, *Listeria monocytogenes* and *L. ivanovii* are pathogenic to humans and animals. Human encephalitis, gastroenteritis, arthritis, conjunctivitis, abortion, and other conditions are produced by *L. monocytogenes* in immunocompromised humans, such as elderly ones and pregnant women [6]. Diverse features of clinical illnesses are also caused by *L. monocytogenes* in animals. Listeriosis in ruminants exhibits encephalitis with the usual "circling" symptoms, meningitis, abortion (in the third trimester), sepsis, stillbirth, and conjunctivitis [7]. This microorganism is extensively found in the natural world and typically infects humans through contaminated food and water. There haven't been any significant outbreaks linked to fish or fish products, despite multiple reports suggesting that they

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can regularly be contaminated with *L. monocytogenes* [8]. In almost 99% of cases, contaminated food and water are the source of *Listeria* spread [9]. Fish containing *L. monocytogenes* are probably from contaminated streams or were handled and processed using contaminated tools and surroundings [10]. Around the world, the bacterium has been widely incriminated as a carrier of infection through fish and fish products [11, 12, 13]. Although *L. innocua* is non-pathogenic, it is isolated often from raw fish samples, in conjunction with *L. monocytogenes* [14]. Both *L. monocytogenes* and *innocua* originated from common ancestors and some strains of *L. innocua* retain virulence factors of *L. monocytogenes* [15]. *L. monocytogenes* can be distinguished from closely related non-pathogenic *L. innocua* using the traditional culture method that requires 6-10 days to get a definitive result. Characteristics generally used for *L. innocua* differentiation over *L. monocytogenes,* based on its failure to cause β-hemolysis, negative CAMP test, and the negative phosphatidylinositol phospholipase C (PI-PLC) activity [16]. Some *L. monocytogenes* strains detected from different processed or natural food showed no haemolysis which makes species identification difficult because only a positive reaction to rhamnose fermentation is then only a differentiating test by the biochemical test methods. Further, several *Listeria* species (*L. monocytogenes*, *L. welshimeri*, *L. innocua*, and *L. grayi ssp. murray*) ferments rhamnose and not xylose. Molecular methods like PCR developed and used for the differentiation of common *Listeria* species were found more rapid, confirmatory, and sensitive [17, 18].

In India, the occurrence of *L. monocytogenes* was investigated at different locations mostly from raw fresh fish samples [13, 14], seafood handling and processing environments [19], and tropical seafood [20, 21] but the information is limited to West Bengal [22]. So, this study was designed for the molecular detection of *L. monocytogenes* and *L. innocua* from fish skin swabs of retail fish markets in Kolkata and South 24 Parganas.

MATERIALS AND METHODS

Source of samples

Two hundred and thirty-four (234) skin swab samples of freshwater rohu fish (*Labeo rohita*) were acquired from July 2023 to January 2024 from different retail fish markets of Patipukur, Ashubabu Bazar in Kolkata (n=165) and Nepalganj, Bakewar in South 24 Parganas (n=69) districts of West Bengal. Then swabs were moistened in a sterile sample collection vial containing 0.9% normal saline solution. The collected samples were preserved under ice cover and transported to the laboratory for processing within 4 hours.

Culture of *Listeria* spp.

ISO: 11290 methods were followed for *Listeria* spp. isolation with two-step selective enrichments. Briefly, pre-enrichment of collected samples was performed using the Fraser broth (half-strength, HiMedia, India) at 30°C for 24 hrs. The second enrichment in the Fraser broth (full-strength, HiMedia, India) was at 37°C for 48 hrs (Bacteriological incubator, Digitech Systems, India) [20]. After this, one loopful of the sample was streaked on the PALCAM agar (HiMedia, India) and incubated at 37°C again for 48 hrs. The presumptive identification of *Listeria* spp. strains was done by observing the grayish-green colonies surrounded by diffuse black zones. Isolates were preserved in Brain heart infusion (BHI) agar (HiMedia, India) slants for further study. These were stained by Gram's staining method and for biochemical identification several tests like catalase, oxidase, Voges Proskauer, nitrate reduction, methyl red, and sugar fermentation of D-xylose, D-mannitol, L-rhamnose,α-methyl D-Mannoside were performed.

Molecular detection of *Listeria* spp. isolates

Bacterial DNA extraction

A crude bacterial DNA template was prepared as per Chen *et al.* [23] with little modification. All presumptive *Listeria* spp. were infused into 2ml BHI broth (HiMedia, India) and incubated at 37°C, overnight. Organisms from the broth were centrifuged (Hermile, Germany) for concentrating at 12000 x g for 3 min. The packed bacterial cells were rediluted in 150µl of nuclease-free water (GCC Biotech, India) followed by boiling for 10 min in a water bath (100^0C) with immediate chilling in ice for cell lysis. The cell suspension was centrifuged at 12000 x g for 10 min for pelleting. The clear supernatant was used as the DNA template (2-5 µL) for PCR reaction. DNA extracted from *L. monocytogenes* strain MTTC-1143 used in this study as a positive control for PCR amplifications was purchased from the Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology, Chandigarh, India.

*L. monocytogenes lmo*1030 gene PCR

All phenotypically positive isolates were further tested for identification of the *Listeria* species-specific *prs* gene and *L. monocytogenes*-specific *lmo*1030 gene by specific PCR as described by Ryu *et al.* [18]. Primer

Table 1. Details of genes and primers used in PCR to detect the *Listeria* spp.

SL No.	Gene	Primer	Sequence	Annealing (°C)	Product size (bp)	Target Species
1.	*prs*	Prs-F	GCTGAAGAGATTGCGAAAGAAG	57.3	370	All *Listeria* spp.
		Prs-R	CAAAGAAACCTTGGATTTGCGG			
	lmo1030	lmo1030-F	GCTTGTATTCACTTGGATTTGTCTGG		509	*L. monocytogenes*
		lmo1030-R	ACCATCCGCATATCTCAGCCAACT			
2.	*lin0464*	lin0464-F	CGCATTTATCGCCAAAACTC	60	749	*L. innocua*
		lin0464-R	TCGTGACATAGACGCGATTG			

sequences were custom synthesized by Bioserve, Hyderabad as detailed in Table 1. PCR was standardized in a thermocycler (T-100, BioRad) with 25 µl PCR reaction mix having 5X Green GoTaq Flexi Buffer 5 µl, (Promega, USA),0.25 µl of 10 mM dNTP mix (Promega, USA), 25nM $MgCl_2$- 2µl (Promega, USA), 1 µl of each primer [*prs* (10 pmol/µl) and *lmo1030* (20 pmol/µl)], and Taq DNA polymerase (5 U/µl) 0.2 µl(Promega, USA), 5µl of template DNA (50-100ng) samples and nuclease-free water up to 25µl. The amplification conditions were standardized as per Ryu et al. [18] except for annealing at 57.3°C for 30s. The PCR end products were detected by gel doc system (Labnet, USA) after electrophoresis in 1.5% (w/v) agarose (SRL, India) gel with 0.5 microgram per ml ethidium bromide (SRL, India).

L. innocua lin0464 gene PCR

All phenotypically *Listeria* sp. positive isolates were again screened for the presence of the *lin*0464 gene (Table 1) by PCR as per Liu *et al*.[16] with minor modifications[24].Target DNA was amplified using a thermocycler (T-100, BioRad) with 25 µl reaction mixture containing 5µl Green GoTaq Flexi Buffer [5X] (Promega, USA),0.5 µl of 10 mM dNTP mix (Promega, USA), 2µl of 25 mM $MgCl_2$ (Promega, USA), 0.5µl (2.5 U/µl) of Taq DNA polymerase (GCC Biotech, India), 1 µl of each primer (0.5µM, Bioserve, Hyderabad), and 5 µl of template DNA(50-100ng) samples of *Listeria* sp.and nuclease-free water up to 25µl. The nuclease-free water and reaction mixture were used as the negative control. The amplification conditions of the PCR cycle were used as per Panera-Martínez *et al.* [24]. The produced DNA products were detected by a gel doc. system (Labnet, USA) using electrophoresis in 1.5% (w/v) agarose (SRL, India) gel having ethidium bromide (0.5 µg/ml) (SRL, India).

RESULTS AND DISCUSSION

Listeria spp. was 44.44% in isolates from 234 swab samples by cultural characteristics and biochemical tests. All the isolates showed morphology characteristics for *Listeria* spp., with the greyish black colony in PALCAM agar (Fig. 1). The isolates appeared as Gram-positive, small, rod-shaped organisms in Gram's staining, and showed typical biochemical reactions. Both *L. monocytogenes* and *L.innocua* isolates were L-Rhamnose and α-Methyl D-Mannoside positive while they were negative to D - Xylose and D - Mannitol in sugar utilization reaction. Out of 104 *Listeria* spp., 82 (49.69%, 82/165) isolates were from retail markets of the Kolkata district and 22 (31.88%, 22/69) were isolated from retail markets of South 24 Parganas district of West Bengal.

Six (2.56%) and 11 (4.7%) isolates from Kolkata were identified as *L. monocytogenes* (Fig. 2) and *L. innocua* (Fig. 3) respectively by species-specific PCR (Table 2) and the rest 87 were *Listeria* sp. as detected by *prs* gene PCR (data not shown). All *Listeria* spp. from samples of South 24 Parganas district were negative to PCR that was definite for the *L. monocytogenes* and *innocua.*This might be due to the difference in the number of samples analyzed in the present study from the South 24 Parganas district.

Over the past few decades, sporadic occurrences of foodborne listeriosis linked to seafood have been documented all over the world. In India, fewer reports are available on the investigations of *Listeria* spp. in the major fish catchment areas due to a lack of easier convenient immunodiagnostic techniques. With the advancement of globalized trade, ensuring seafood quality is very crucial in food export in India to get acceptance in global markets and generate healthy profits [25]. As per the available literature, there is no reported study to compare *L. monocytogenes* detection from freshwater fish rohu (*Labeo rohita*) in West Bengal [26]. However, a recent report showed positivity for *Listeria* spp. in *L. rohita* samples collected from wholesale fish market at Baghajatin and retail fish markets at Garia in Kolkata using HiFast™ food pathogen detection kit (HiMedia, India) [27]. Prevalence

Table 2. Molecular detection of *Listeria* isolates by species-specific PCR.

Isolate No.	Region	*prs* gene PCR	*lmo1030*gene PCR	*lin0464* gene PCR
FS73	Kolkata	+	-	+
FS96	Kolkata	+	-	+
FS97	Kolkata	+	-	+
FS98	Kolkata	+	-	+
FS101	Kolkata	+	-	+
FS102	Kolkata	+	-	+
FS168	Kolkata	+	-	+
FS171	Kolkata	+	-	+
FS172	Kolkata	+	-	+
FS217	Kolkata	+	+	-
FS218	Kolkata	+	+	-
FS219	Kolkata	+	+	-
FS220	Kolkata	+	+	-
FS221	Kolkata	+	+	-
FS222	Kolkata	+	+	-
FS223	Kolkata	+	-	+
FS224	Kolkata	+	-	+

Fig. 1. *L. monocytogenes* strain (FS218) colonies in PALCAM agar plate (greyish black colony).

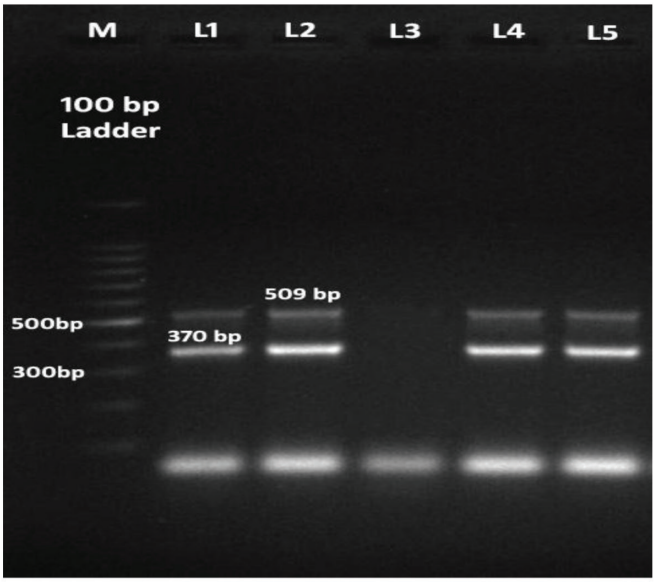

Fig. 2. PCR amplification of *prs* and *lmo*1030 genes in *L. monocytogenes* strains. [Lane M: DNA Ladder (100 bp), Lane L1: Positive control, L3: Negative control, L2, L4, L5: Test samples FS217, FS218, FS222].

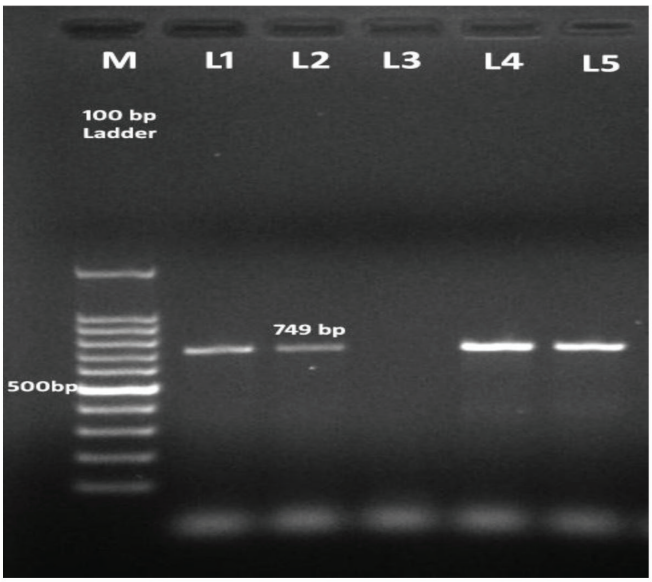

Fig. 3. Gel electrophoresis of PCR products of *lin0464* gene in *L. innocua* strains. [Lane M: DNA Ladder (100 bp), Lane L1, L2, L4, and L5:Test samples FS96, FS102, FS168, FS224 correspondingly;L3: Negative control].

of *Listeria* spp. (19.5%, 39/200) was much lower from the muscles and viscera of freshwater fish and walking catfish (*Clarias batrachus*) in Nagpur, Maharashtra than the present study [28]. The majority were *L. monocytogenes* (26, 67%), followed by *L. seeligeri* (8, 21%), *L. welshimeri* (2, 5%), *L. grayi*(3, 8%), and no *L. innocua* isolate was detected. Similar to the present study, from raw fresh fish samples, *L. monocytogenes* (1.3 %) was found low in prevalence than *L.* innocua (30.8%) from Mangalore, India [14]. *L. monocytogenes* positivity of 2.31% (27/1165) isolates were also reported in Northeast India from five different freshwater fish species *viz*. Magur (*Clarius batrachus*), Hingi (*Heteropneustes fossilis*), Tengra (*Mystus seenghala*), Koi (*Anabas testudineus*) and Botia (*Lepidocephalus guntea*). The highest frequencies of positive isolates, 12

(1.03%) were from samples of intestines followed by 10 (0.85%) from gills and 5 (0.43%) from flesh samples of freshwater fish [13]. *Listeria* spp. was present in 32.3% of fresh fish samples, 27.1% of frozen fish samples, and 5% of dry fish samples in Kerala [20]. Their investigations also suggested that the presence of overall *Listeria* spp. along with *L. monocytogenes* (1.2%) was comparatively lower than the present study except for *L. innocua* (41.7%). *Listeria* spp were also detected in 24.35% (28/115) of raw/fresh seafood samples in Goa, of which 8.7 % (10/115) and 15.65% (18/115) isolates were accounted for *L. monocytogenes* and *innocua,* correspondingly [21]. In the coastal city of Mysore, *Listeria* sp. was isolated in 62 fresh fish samples (37.8%), *L. monocytogenes* from 3 (1.83%) samples which were nearly similar to this study with a higher frequency of *L. innocua* was found from 50 isolates (30.49%) [29]. Likewise, a study in Kerala investigated 227 samples from retail fish markets and detected 20% of *Listeria* spp. Among them, the occurrence of *L. innocua* was more (17.2%) than *monocytogenes* (2.7%) [30]. *L. monocytogenes* was also detected in two fish samples (2.0%) in Kashmir [31]. These reports indicated that raw/fresh fish and seafood are often infected with these bacteria at low percentages. Competitive exclusion of *L. monocytogenes* was reported from samples using the conventional culture method when *L. innocua* was present due to the lesser generation time of the latter [32]. *L. innocua* being more frequent in samples, it can be regarded as an indicator bacterium for studying *L. monocytogenes* [15]. *L. monocytogenes* have the potential to cause public health problems if food safety standards are not followed strictly.

Although considered non-pathogenic, *L. innocua* also rarely causes human infections [33]. From the past research, it appears that there is scanty data on the pathogenicity of *Listeria innocua.* Rarely *L. innocua* strains are associated with cerebral listeriosis in bulls [34] and abortions in cattle [35]. *L. innocua* and *L. monocytogenes* are related genetically and some special *L. innocua* strains also possess listeria pathogenicity island-1 and 3, other virulence genes similar to *L. monocytogenes* [36, 37]. Similar morphological, biochemical, and serological characteristics made the differential identification from *L. monocytogenes* erroneous, therefore the PCR technique based on a unique *lin0464* gene for *L. innocua* was found to be very useful and rapid [23]. Besides, *L. innocua* strains have been reported to be reservoirs of resistance genes for antibiotics and the prevalence of these bacteria is of special significance in the emergence and transfer of antimicrobial resistance of *L. monocytogenes* [38]. Their coexistence in similar environmental conditions may be helpful for antibiotic resistance or virulence gene transfer. Being ubiquitous and highly adaptable, *L. monocytogenes* and *L. innocua*-like food-borne pathogen scrutiny programs is useful to appreciate the source of infection, virulence, and antimicrobial resistance [39].

CONCLUSION

A low occurrence of pathogenic *Listeria monocytogenes* and avirulent *L. innocua* was reported for the first time in freshwater fish rohu (*L. rohita*) samples in retail markets of West Bengal. This warrants attention for public health, particularly for risk in immunocompromised people, babies, elderly individuals, and pregnant women. Due to the close genetic interconnection of the *Listeria* species and the presence of atypical strains, all conventional species identification methods have limitations in differentiating species. The lack of simple tests based on immunodiagnostics is a major drawback in identifying foodborne *Listeria* spp. and animal listeriosis investigations remain under-reported. Molecular methods based on specific marker genes for distinguishing *Listeria* spp. from the pathogenic *L. monocytogenes* offer rapid confirmatory detection and should be widely employed in microbiological surveillance of major foodborne pathogens.

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